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Award Number: DAMD17-01-1-0252

TITLE: An Epithelial-Derived, Integral Membrane, Kunitz-Type Serine Protease Inhibitor in Breast Cancer

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REPORT DATE: August 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Annual Summary (1 Aug 2002 - 31 Jul 2003)

4. TITLE AND SUBTITLE
An Epithelial-Derived, Integral Membrane, Kunitz-Type Serine Protease Inhibitor in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0252

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

In the current research plan, we proposed to study the anti-tumor and anti-protease activity of a membrane-bound Kunitz-type serine protease inhibitor (KSPI; also known as HAI-1). In order to investigate how HAI-1 regulates matriptase function, we have constructed mutants of the HAI-1 proteins, including point mutations at the active center of both Kunitz domains and the calcium cage of the LDL receptor class A domain, and the deletion of entire LDL receptor class A domain. Using wild-type HAI-1 and these mutants, we first discovered that HAI-1 can help matriptase traffic out of ER and Golgi apparatus. The functional domain of HAI-1 to facilitate matriptase trafficking was identified to be Kunitz domain I, but not domain II. Interestingly, HAI-1 is aslo required for matriptase activation. The intact LDL receptor class domain A of HAI-1 is required for matriptase activation. Taken together, in additional to functioning as an inhibitor for matriptase, HAI-1 is also required for matriptase trafficking and activation.

14. SUBJECT TERMS
Anti-tumor, protease, protease inhibitor, cancer invasion HGF activation, pro-uPA activation, FGF

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

The death of women with breast carcinoma mainly results from metastasis. Metastatic breast cancer cells must escape from a primary tumor and migrate through anatomical barriers in order to gain access to the blood or lymphatic system and establish at a new site in the body. Cellular motility and degradation of extracellular matrix (ECM) are two of the major events in breast cancer metastasis and can be promoted by stromal-derived, ECM-degrading protease systems, such as the urokinase type plasminogen activator (uPA) system and by motility factors, such as hepatocyte growth factor (HGF)/scatter factor (SF). In order to understand how breast cancer cells regulate both stromal-derived, ECM degradation and cellular motility for metastasis, we have discovered and characterized in human breast cancer a new epithelial-derived, type 2 integral membrane, serine protease, matriptase, and its cognate inhibitor, a Kunitz-type serine protease (KSPI), a type 1 integral membrane protein which was initially identified as an inhibitor of hepatocyte growth factor activator and named as HAI-1 (1-3). Both matriptase and HAI-1 have been implicated in the regulation of ECM-degradation of cellular motility (4). In the current research plan, we proposed to study the anti-tumor and anti-protease activity of this membrane-bound Kunitz inhibitor.

Body:

During the August 02-July 03 period we had addressed Aim 2 of the original proposal:

Year 2: In the second year, we will continue our structural analysis of KSPI by expression of KSPI mutants in a Baculovirus expression system, purification of these mutants, and characterization of their inhibition kinetics (Aim 2). In addition, we will carry out transfection of these KSPI mutants into α18 and MDA MB-435 breast cancer cells (Aim 2).

In the second year, we have finished the structure analysis of KSPI, now termed HAI-1. The transfection of HAI-1 in breast cancer cells have been completed and reported in the annual report of last year. Thus, we focus on the structural and functional analysis of HAI-1. Because the inhibition kinetics of both Kunitz domains of HAI-1 was published by others (5), we took an alternative approach to investigate how HAI-1 regulates matriptase functions. We constructed mutants of the HAI-1 protein, to test the role of the Kunitz and LDL-receptor class A domains of the inhibitor in the trafficking and activation of matriptase (Fig. 1). HAI-1 protein was mutated by site-directed or deletion mutagenesis to create mutant proteins that were used in transfection experiments. Site-directed mutations of HAI-1 included replacement Arg-258 and Arg-260 residues with Leu in Kunitz domain I (R258L and R260L HAI-1), and Lys-385 with Leu in Kunitz domain II (K385L HAI-1). A point mutation of the LDL-receptor class A domain in the putative Ca^{2+} binding cage (D349Y HAI-1) was created by replacement of Asp-349 with Tyr, and the entire domain was also deleted (HAI-1 Δ325-355).

Co-expression of HAI-1 with matriptase dramatically enhanced the expression of matriptase by facilitating its intracellular trafficking, permitting subsequent trans-activation of the protease

In a previous study, we showed that breast cancer cells constitutively activate matriptase (6). Thus, we chose BT549 human breast cancer cells to investigate matriptase activation. Because BT549 human breast cancer cells do not endogenously express either matriptase or HAI-1, as assessed by anti-matriptase or anti-HAI-1 western blotting (Fig. 2A and C), detection of transfected matriptase and HAI-1 could not be confused with endogenous proteins. When BT549 cells were transiently transfected with wild type matriptase, only a very low level of matriptase protein was detected by western blotting using the anti-matriptase mAb M32 that recognizes total (one-chain latent plus two-chain activated) matriptase upon long exposure of blots (Fig. 2A). Immunofluoresence staining using Texas red conjugated phalloidin (actin) together with the anti-matriptase mAb M32 (Fig. 3A) suggested that the poor expression could be due to a defect in intracellular trafficking of the protease. Indeed, immunofluorescence using an antibody that recognizes GM130, a Gogi-specific marker, together with Alexa-Fluor®594-conjugated M32 matriptase-specific antibody (Fig. 3D), revealed that matriptase accumulated in a cellular location consistent with the endoplasmic reticulum and Golgi apparatus. To investigate whether this defect in trafficking may be due to the unregulated proteolytic activity of matriptase,

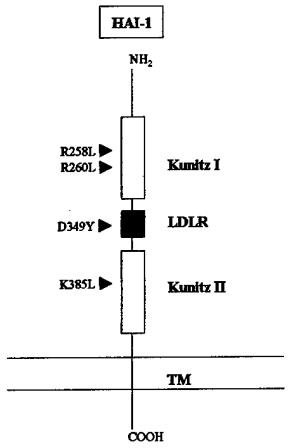

Figure 1. Mutations in HAI-1 used to assess the function of individual domains and motifs in matriptase trafficking and activation.

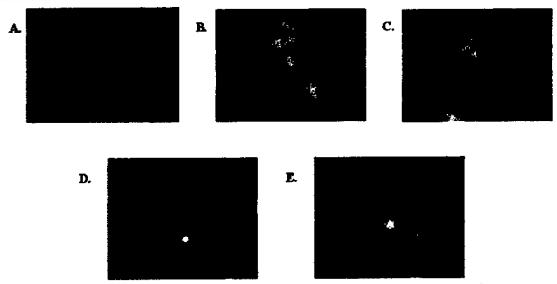

Figure 3. The poor expression of wild-type matriptase in BT549 cells is due to a defect in matriptase intracellular trafficking. BT549 breast cancer cells were transfected with matriptase alone (A), matriptase plus HAI-1 (B), or with the catalytically inactive triad mutant S805A matriptase (C) and stained for matriptase using the M32 mAb and FITC-labeled rabbit anti-mouse IgG (green) and for actin using Texas red conjugated phalloidin (red) as described in Materials and Methods. To demonstrate more clearly the site of the matriptase trafficking defect, matriptase was transfected alone (D) or with HAI-1 (E), and cells were stained for matriptase using Alexa Fluor®594-conjugated M32 mAb (red), for the Golgi-specific protein GM130 using a GM130-specific mAb and FITC-conjugated rabbit anti-mouse IgG (green), and for nuclei using DAPI (blue). Data is representative of three independent experiments.

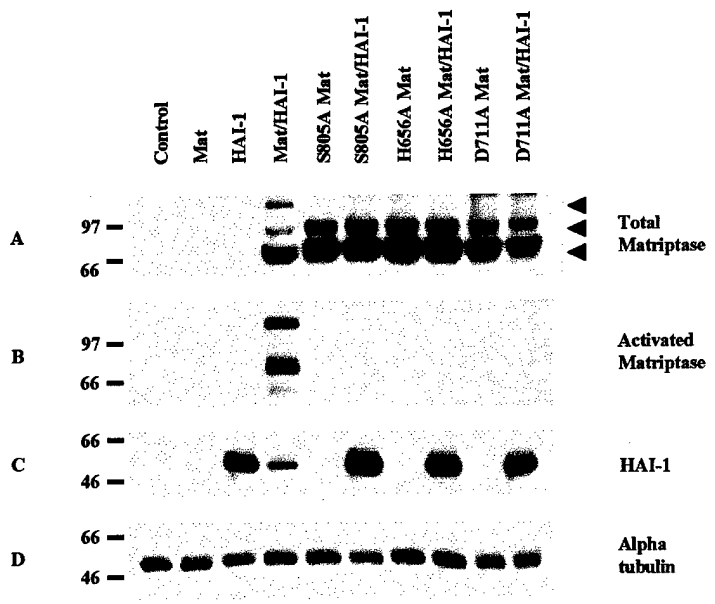

Figure 2. Co-transfection of HAI-1 with matriptase, or transfection of matriptase catalytic triad mutants, enhanced the protein expression of matriptase in BT549 cells. BT549 breast cancer cells were transfected with control pcDNA3.1 vector (Control), matriptase cDNA (Mat), HAI-1 cDNA (HAI-1), both matriptase and HAI-1 cDNAs simultaneously (Mat/HAI-1), or with catalytic triad mutants (S805A Matriptase, H656A Matriptase, and D711A Matriptase) alone or with HAI-1 as indicated. Total matriptase was detected by western blotting with the M32 mAb that recognizes total (latent plus activated) matriptase (A). Activated matriptase was detected by using the M69 mAb that recognizes only activated protease (B). Activated protease could also be detected by formation of the 120 kDa matriptase:HAI-1 complex seen in panel A. HAI-1 transfection was demonstrated by western blotting using the M19 mAb (C), and equal protein loading was confirmed by using an alpha tubulin-specific mAb (D). Data is representative of three independent experiments. Arrowheads indicate the position of the 120 kDa matriptase:HAI-1 complex (top arrowhead), the 95 kDa full-length matriptase (middle arrowhead), and the 70kDa amino-terminal processed form of matriptase (bottom arrowhead). A low level of matriptase in the absence of co-transfected HAI-1 (Mat) could be detected by M32 western blotting upon longer exposure of the blot.

the inhibitor HAI-1 was co-transfected with matriptase. HAI-1 corrected the defect in intracellular trafficking and resulted in much improved expression of the protease, as shown by western blotting and immunofluorescence using the anti-matriptase mAb M32 (Fig. 2A, and Fig. 3B and E). In addition, mutants of matriptase altered in the catalytic triad (S805A, H656A, and D711A matriptase) were able to traffic in the absence of HAI-1 (Fig 3C), and were thus expressed at levels comparable to wild-type matriptase co-expressed with HAI-1, as demonstrated by matriptase western blotting (Fig 2A). Identical results were found for a matriptase mutant altered at Arg-799 (R799A matriptase) in the substrate-binding pocket (data not shown).

In cells transfected with both wild-type matriptase and HAI-1, both the uncomplexed, processed form of matriptase (70kDa form), and the processed form complexed to HAI-1 (120kDa complex), were observed, in addition to the full-length matriptase that migrates at approximately 95kDa (Fig 2A). This result indicates that matriptase had been activated when co-transfected with HAI-1, since only the activated form of the enzyme binds to the inhibitor HAI-1 to form the 120 kDa complex. In addition, we confirmed this observation by western blotting with the M69 mAb that recognizes only the two-chain, activated form of matriptase, and not the one-chain, latent form of the protease (Fig 2B). It should be pointed out that both the complexed and uncomplexed forms of matriptase were observed in M69 western blots, and the ratio of these forms varies from experiment to experiment for unknown reasons (6; 7; 8). When mutants of matriptase that had catalytic triad defects were expressed alone or with HAI-1, the activated form of the protease was not observed, as indicated by a lack of the formation of a 120 kDa matriptase/HAI-1 complex and lack of immunoreactivity with the M69 mAb (Fig 2A and B). This may indicate that matriptase activation occurs by a trans-activation mechanism in BT549 breast cancer cells, and not likely due to the activity of other proteases.

The proper trafficking and enhanced expression of the matriptase protein when co-transfected with HAI-1 is not a phenomena limited to BT549 cells, but was also seen when matriptase and HAI-1 were transfected into MDA MB-231 and MDA MB-435 cancer cells (data not shown). These two cancer cell lines also do not endogenously express matriptase or HAI-1 (9).

The first Kunitz domain, but not the second, is required for HAI-1 to facilitate matriptase intracellular trafficking, and the LDL receptor A domain of HAI-1 is essential for matriptase activation.

Since the intracellular trafficking of matriptase depended upon HAI-1, we were able to test which domains in HAI-1 were essential for protease trafficking. HAI-1 contains two Kunitz-type serine protease inhibitory domains, Kunitz domain I (at the amino terminus) and Kunitz domain II (at the carboxyl terminus), with an intervening LDL receptor class A domain (Fig. 1). In addition to the trafficking of matriptase, it is of interest to determine whether these three HAI-1 domains participate in the activation of the protease.

The Kunitz domain is an approximately 60 amino acid long serine protease inhibitory domain for which the bovine basic pancreatic trypsin inhibitor (BPTI) represents the prototype structure. The P1 residue of Kunitz-type inhibitory domains (the amino acid residue C-terminal to the second conserved cysteine residue) is recognized as the active center responsible for the inhibitory specificity. For example, the corresponding amino acid residues in the Kunitz domains of HAI-1 are Arg-260 in domain I and Lys-385 in domain II, and therefore these Kunitz domains are predicted to be specific for trypsin-like serine proteases such as matriptase (interacting with Asp-799 in the substrate binding pocket of matriptase). Mutation of this residue will completely abolish the inhibitory activity of a Kunitz domain. Previously, molecular modeling strongly suggested that the first, but not the second, Kunitz domain of HAI-1 is responsible for the inhibition of matriptase (10). In addition to Arg-260, Arg-258 was also suggested to be crucial for the activity of Kunitz domain I. Therefore, we constructed point mutations at these three critical basic residues in Kunitz domains I and II of HAI-1 (Arg-258, Arg-260, and Lys-385, see Fig.1).

Mutation of critical arginine residues in the first Kunitz domain of HAI-1 (R258L and R260L HAI-1) completely abolished the ability of HAI-1 to facilitate the intracellular trafficking of matriptase (Fig. 4A). In co-transfection experiments with these HAI-1 mutants, matriptase was poorly expressed, as determined by M32 western blotting (Fig. 4A), and did not traffic properly as determined by matriptase immunofluorescence (data not shown). Mutation of a critical basic residue in the second Kunitz domain of HAI-1 (K385L HAI-1), however, did not affect the ability of HAI-1 to facilitate matriptase trafficking, since matriptase expressed well

(Fig. 4A) and underwent proper intracellular trafficking, as confirmed by matriptase immunofluorescence (data not shown). Western blotting with the activation-specific M69 mAb showed that matriptase was activated when it was co-transfected with wild-type HAI-1 or with the K385L HAI-1 mutant that facilitated its proper trafficking (Fig. 4B). The activation of matriptase when co-transfected with the Kunitz domain I mutants of HAI-1 that failed to facilitate matriptase trafficking could not be assessed, as they were barely detectable by M32 matripase western blotting. It should be noted that the R258L and R260L HAI-1 mutants did not express well when transfected with matriptase, in contrast to the K385L HAI-1 mutant (Fig. 4C). However, this was due to the defect in intracellular trafficking created by unopposed matriptase activity, and not by poor transfection efficiency (Fig 5). These results, together with the fact that the transmembrane protein E-cadherin does not traffic properly when co-expressed with matriptase in the absence of HAI-1 (data not shown), suggest that matriptase proteolytic activity generally interferes with the trafficking of transmembrane proteins when transfected in the absence of HAI-1.

In addition to exploring the importance of the Kunitz domains in matriptase trafficking, we examined the role played by the HAI-1 LDL receptor class A domain in trafficking and activation. The LDL receptor class A domain is an approximately 40 amino acid long structure with three pairs of disulfide linkages (11), and is found in membrane receptors and some proteases. The crystal structure of the fifth LDL receptor class A domain in the LDL receptor revealed that the domain contains six amino acids that coordinate a Ca^{2+} atom in an octahedral arrangement, termed the calcium cage (12). Point mutation at critical residues in the calcium cage potently inhibits ligand binding to this domain (13). Therefore, we constructed a point mutation at a critical Asp residue involved in calcium coordination in the calcium cage of the LDL receptor class A domain of HAI-1 (D349Y HAI-1) to investigate the function of this domain in matriptase trafficking and activation (Fig. 1). We also created another mutant that contains a deletion of the entire LDL receptor class A domain (HAI-1 ΔLDLR). Neither of these mutants of HAI-1 affected the ability of the inhibitor to facilitate matriptase intracellular trafficking (Fig. 4A), indicating that the LDL receptor class A domain is not essential for matriptase trafficking. However, loss of the LDL receptor class A domain function through point or deletion mutation dramatically inhibited the activation of co-transfected wild-type matriptase, as demonstrated by a lack of formation of the matriptase/HAI-1 complex (Fig 4A) and by the lack of M69 mAb immunoreactivity (Fig. 4B).

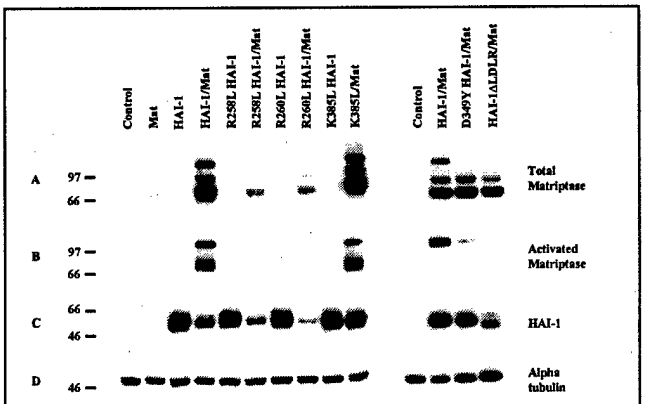

Figure 4. The facilitation of matriptase trafficking by HAI-1 requires HAI-1 Kunitz domain I but not Kunitz domain II, and the HAI-1 LDLR class A domain is required for matriptase activation. BT549 breast cancer cells were transfected with control pcDNA3.1 vector (Control), matriptase cDNA (Mat), HAI-1 cDNA (HAI-1), both matriptase and HAI-1 cDNAs simultaneously (Mat/HAI-1), or with HAI-1 mutants (R258L HAI-1, R260L HAI-1, K385L HAI-1, D349Y HAI-1, HAI-1ΔLDLR) transfected with or without wild-type matriptase as indicated. Total matriptase was detected by western blotting with the M32 mAb that recognizes total (latent plus activated) matriptase (A). Activated matriptase was detected by using the M69 mAb that recognizes only activated protease (B). Activated protease could also be detected as a 120 kDa matriptase:HAI-1 complex seen in panel A. HAI-1 transfection was demonstrated by western blotting using the M19 mAb (C), and equal protein loading was confirmed by using an alpha tubulin-specific mAb (D). Data is representative of three independent experiments.

Key research accomplishments:

- We have constructed mutants of the HAI-1 proteins
- HAI-1 is required for matriptase trafficking out of ER and Golgi apparatus
- The proteolytic activity of matriptase is toxic to cells when the protease was overexpressed in breast cancer cells in the absence of HAI-1.

- HAI-1 can help matriptase traffic out of ER and Golgi apparatus.
- The functional domain of HAI-1 to facilitate matriptase trafficking is Kunitz domain I, but not domain II.
- HAI-1 is required for matriptase activation, in addition to its role in matriptase trafficking.
- The LDL receptor class domain A is the functional domain for its role inmatriptase activation.

Reportable outcomes:

1. Williams, C., Benaud, C., Oberst, M.D., Dickson, R.B., and Lin, C.-Y., (2002) Deregulation of matriptase activity in breast cancer cells. Proteases, Extracellular Matrix, and Cancer. AACR-Special Conference in Cancer Research, Hilton Head Island, South Carolina
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Conclusion:

In order to investigate how HAI-1 regulates matriptase function, we have constructed mutants of the HAI-1 proteins, including point mutations at the active center of both Kunitz domains and the calcium cage of the LDL receptor class A domain, and the deletion of entire LDL receptor class A domain. Using wild-type HAI-1 and these mutants, we first discovered that HAI-1 can help matriptase traffic out of ER and Golgi apparatus. The functional domain of HAI-1 to facilitate matriptase trafficking was identified to be Kunitz domain I, but not domain II. Interestingly, HAI-1 is aslo required for matriptase activation. The intact LDL receptor class domain A of HAI-1 is required for matriptase activation. Taken together, in additional to functioning as an inhibitor for matriptase, HAI-1 is also required for matriptase trafficking and activation.

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Appendices: None